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Improved enzyme production by co-cultivation of *Aspergillus niger* and *Aspergillus oryzae* and with other fungi

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ABSTRACT

Aspergillus niger and *Aspergillus oryzae* were co-cultivated with each other and with *Magnaporthe grisea* or *Phanerochaete chrysosporium*, respectively. Enzyme assays for plant polysaccharide and lignin-degrading enzymes showed that co-cultivation can improve extracellular enzyme production. Highest β-glucosidase, α-cellobiohydrolase, β-galactosidase, and laccase activities were found for *A. oryzae* in combination with other fungi, in particular with *P. chrysosporium*. Highest β-xylosidase activity was obtained when *A. niger* was co-cultivated with *P. chrysosporium*. SDS-PAGE protein profiles demonstrated that *A. niger* and *A. oryzae* contributed most to the overall enzyme activities found in the culture medium of the mixed cultivations. These data demonstrate that co-cultivation of two major industrial fungi, *A. niger* and *A. oryzae*, results in improved production of biotechnologically relevant enzymes.

1. Introduction

Aspergillus is a genus of filamentous fungi representing 838 epithets (http://www.indexfungorum.org/Names/Names.asp). *Aspergillus niger* and *Aspergillus oryzae* are the most commonly used industrial *Aspergillus* species for the production of pharmaceuticals, food ingredients and enzymes (Berka et al., 1992; Pandey et al., 1999). *A. niger* and *A. oryzae* produce a broad range of enzymes related to degradation of plant polysaccharides, such as cellulose, xylan, xyloglucan, galactomannan and pectin (de Vries and Visser, 2001). These enzymes are essential to convert the natural carbon sources of these fungi (mainly plant polymers) into small molecules that can be taken up into the cell.

During their natural life cycle most fungi encounter other microorganisms and live in mixed communities with complex interactions, such as symbiosis or competition. It is known that a particular biotope may be colonized by several Aspergilli. For instance, *Aspergillus flavus, Aspergillus japonicus*, *A. niger, Aspergillus parasiticus, Aspergillus ustus* and *Aspergillus wentii* can be isolated from tombs and mummies (Arya et al., 2001). Pre-digestions of the carbon source (e.g. plant-derived polysaccharides) occur extracellularly for fungi, and therefore it is highly likely that natural degradation of plant biomass occurs by mixtures of enzymes produced by several organisms. Co-cultivation of fungi may therefore result in more efficient enzyme mixtures for industrial applications than those obtained from mono-cultivations.

Fungal co-cultivations have been previously described for the production of specific enzymes. *A. niger* and *Trichoderma reesei* were co-cultivated for cellulase production (Maheshwari et al., 1994), while *Aspergillus ellipticus* and *Aspergillus fumigatus* were co-cultivated for cellulase and β-glucosidase production (Gupte and Madamwar, 1997). Co-cultivation of *Pleurotus ostreatus* and *Phanerochaete chrysosporium* resulted in higher production of ligninolytic enzymes (Verma and Madamwar, 2002), which was also found for a *Trametes* and a *Trichoderma* strain (Zhang et al., 2006).

A. niger and *A. oryzae* are two of the most important fungi worldwide for biotechnological applications and both have a long history with respect to strain improvement to optimize enzyme production. They also have a significantly different set of polysaccharide-degrading enzymes in their genome (Coutinho et al., 2009). However, co-cultivation of these two species has not been described. In this study we applied co-cultivation to these two

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species, to see whether this could improve enzyme production. In addition, we performed co-cultivations of these two Aspergilli the ascomycete phytopathogen *Magnaporthe grisea*, and the basidiomycete white rot fungus *P. chrysosporium*, respectively. These fungi have significantly different sets of enzymes based on their genome sequence (see www.cazy.org) as well as significantly different lifestyles and may therefore effect enzyme production differently than the Aspergilli.

2. Materials and methods

2.1. Strains, media and culture conditions

The fungal strains used in this study are *A. niger* N402 (Bos et al., 1988), *A. oryzae* RIB40 (Machida et al., 2005), *M. grisea* Guy-11 (Dean et al., 2005) and *P. chrysosporium* RP78 (Martinez et al., 2004). All strains were grown on malt extract agar (MEA) to obtain spores. Spore suspensions were diluted to 500 spores/μl (5×10^5 spores/ml) for plate growth experiments. To investigate growth rate and mycelial interactions, combinations of fungi were grown on MEA and *Aspergillus* complete medium (CM) (de Vries et al., 2004) with 1% glucose, 3% sugar beet pulp (SBP) or 3% wheat bran (WB). Fungi were co-cultivated by inoculating 2 μl of a spore suspension (5×10^5 spores/ml) with a distance of 3 cm or by mixing the spores and inoculating them in the centre or over the whole surface of the plate. For liquid cultures, fungi were grown in *Aspergillus* minimal medium (MM) (de Vries et al., 2004) + 1% wheat bran. Fungi were inoculated at 5×10^6 spores/ml and incubated at a rotary shaker at 250 RPM. In co-cultivations, half the number of spores from each species was used as compared to the single incubations to maintain a total inoculum of 5×10^6 spores/ml. Cultures were grown at 25 °C (for combinations with *M. grisea*) or 30 °C (for combinations with *P. chrysosporium*). Culture filtrate samples were prepared by harvesting aliquots of the culture and centrifuging them for 10 min at 10.000 RPM. The supernatant was then transferred to a new tube and used as the culture filtrate sample.

2.2. Enzyme assays

Extracellular enzyme assays were carried out as described (de Vries et al., 2004) with some modifications. 100 μl mixture were used for the reaction, including 10 μl culture filtrate, 50 μl of 50 mM sodium acetate buffer (pH5.0), 30 μl water and 10 μl of 0.1% substrate. Substrates used were obtained from Sigma and were *p*-nitrophenol-β-D-glucopyranoside to measure β-glucosidase (BGL) activity, *p*-nitrophenol-D-cellobioside to measure cellobiohydrolase (CBH) activity, *p*-nitrophenol-β-D-xylopyranoside to measure β-xylosidase (BXL) activity, *p*-nitrophenol-α-L-arabinofuranoside to measure α-L-arabinofuranosidase (ABF) activity and *p*-nitrophenol-β-D-galactopyranoside to measure β-galactosidase (LAC) activity. These five enzyme activities were selected because they are commonly produced by fungi and are important for many industrial applications. The mixtures were incubated for 1 h at 30 °C, after which the reaction was stopped with 100 μl of 0.25 M sodium carbonate. The optical density at 405 nm was measured with a model 550 microplate reader (Bio-Rad).

Extracellular laccase activity was measured with 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) as the substrate according to Srinivasan et al. (1995). The reaction mixture (in a total volume of 1 ml) contained 0.7 ml water, 0.1 ml 0.5 M glycine–HCl (pH3.0), 0.1 ml culture filtrate and 0.1 ml 0.14 mM ABTS. The mixtures were incubated for 1 h at 30 °C, after which the optical density was measured at 405 nm.

All assays were performed in triplicate. The standard deviation is determined based on the average value of two duplicate cultures.

Total protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific) to compare overall protein production in the cultures.

2.3. SDS-PAGE profiles

Extracellular proteins were separated by SDS-PAGE using 10% polyacryl amide gels. After fixing the gel with 45% methanol and 10% acetic acid overnight, proteins were stained with silver according to Switzer et al. (1979). The marker for the protein gels is the sigma wide range marker (S8445).

3. Results

3.1. Co-cultivation of fungi

To determine whether the selected fungi were able to grow as mixed cultures, several plate growth experiments were performed as described in Materials and methods. All the 4 fungi had different radial expansion rates on solid culture medium (data not shown). *A. niger* and *A. oryzae* were overall the fastest growing fungi with *A. niger* being the faster of the two, while *M. grisea* in particular grew slowly.

All of the tested fungi grew on the four culture media tested in this study, but CM + 1% glucose and CM + 1% WB resulted in best growth. WB was chosen as the carbon source in liquid culture media for the rest of the study, since an earlier study demonstrated that on this carbon source a broad range of hydrolytic enzymes is produced by several *Aspergillus* species (Meijer et al., in press).

Mixed cultivations were initially performed on plates to determine whether the species were able to grow in the presence of each other to select the fungal species for the liquid cultivations. Selection of the fungi used in this study was based on analysis of their set of polysaccharide-degrading enzymes (www.cazy.org) and their lifestyle. The edges of the colonies of *A. niger*, *A. oryzae* and *M. grisea* would grow through each other in the zone where the colonies would meet when they were inoculated at a distance away from each other, but would not strongly invade the space occupied by the other fungus (Fig. 1A, D, E). *P. chrysosporium* did grow around and through the other fungi, without a significant effect on the growth of the other fungus (Fig. 1B, C). When they were inoculated in the same spot from mixed spores, sectors containing either *A. niger* or *A. oryzae* could be observed on the plates (Fig. 1F). Interestingly, this was different for combinations with *P. chrysosporium* (Fig. 1G–I). No mycelium of this fungus was visible during the first 24 h of cultivation (data not shown). From the second day, a few hyphae appeared, spreading to the whole plate except the space occupied by the other fungi. On the third day, these hyphae spread further covering the plate with a white colony. The hyphae appeared to have grown through the other fungus to reach the outside of the colony.

Preliminary experiments in our lab with combinations of other fungi such as *T. reesei*, *Rhizopus oryzae* and *Gibberella zeae* were also performed. In all these combinations one species appeared to outcompete the other (data not shown) and therefore no further studies were done with these combinations.

3.2. Mixed cultures affect extracellular enzyme production

To determine the influence of fungal co-cultivation on extracellular enzyme production, the selected fungal species were grown alone or as mixed culture for 72 h in MM + 1% wheat bran. Five polysaccharide-active enzymes were selected: β-glucosidase, cellobiohydrolase, β-xylosidase, α-arabinofuranosidase and β-galactosidase as they had previously been shown to be produced

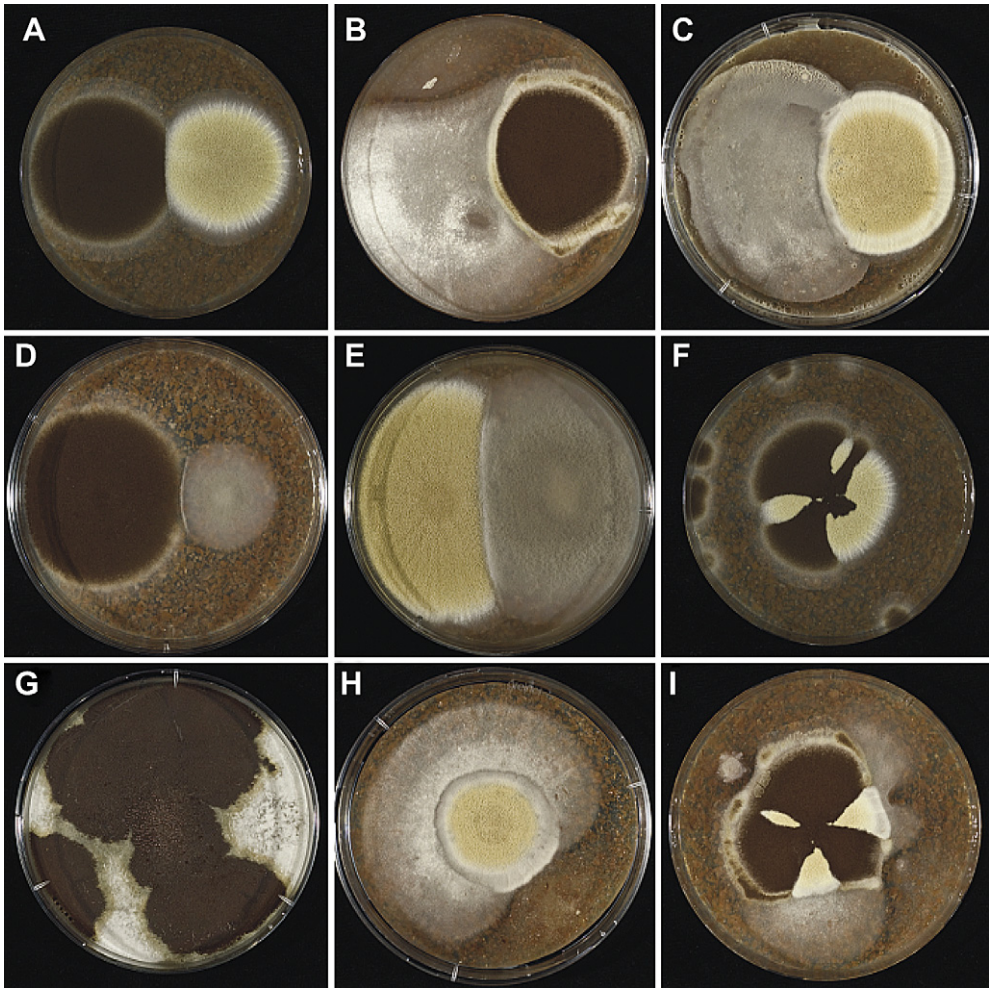

Fig. 1. Co-cultivation of different fungi on solid CM + 3% WB by inoculating them separately (A–E) or as a mixture of spores (F–I). A. *A. niger* + *A. oryzae*; B. *A. niger* + *P. chrysosporium*; C. *A. oryzae* + *P. chrysosporium*; D. *A. niger* + *M. grisea*; E. *A. oryzae* + *M. grisea*; F. *A. niger* + *A. oryzae*; G. *A. niger* + *P. chrysosporium*; H. *A. oryzae* + *P. chrysosporium*; I. *A. niger* + *A. oryzae* + *P. chrysosporium*.

during growth on wheat bran (Meijer et al., in press). In addition, we also measured laccase activity, to determine whether mixed cultivation also affected lignin degradation.

Overall, most mixed cultivations resulted in increased enzyme activities compared to the single cultures, although not always for all enzymes tested (Tables 1 and 2). β-Glucosidase activity was increased for all combinations except *A. niger* + *P. chrysosporium*, while cellobiohydrolase and α-arabinofuranosidase activities were only increased in the combination *A. niger* with *M. grisea*. β-Xylosidase activity was increased in the combinations of *A. niger* + *P. chrysosporium* and *A. oryzae* + *M. grisea*, while the activity in the *A. niger* + *A. oryzae* co-cultivation was approximately the average of the two single cultures. Co-cultivations of *A. oryzae* with either *P. chrysosporium* or *M. grisea* resulted in increased β-galactosidase activities. The presence of *P. chrysosporium* resulted in an increase in laccase activity for both *A. niger* and *A. oryzae*. Surprisingly, *M. grisea* produced very high laccase activity, which was reduced in combinations with *A. niger* and *A. oryzae*.

Total protein production in the cultures was also determined (Table 3). This demonstrated that for *A. niger* and *A. oryzae* protein production at 30 °C was higher than at 25 °C. The mixed cultivation of *A. niger* and *A. oryzae* produced a little less protein than the individual cultures, while combination of either fungus with *P. chrysosporium* resulted in a small increase in protein production. Only the combination of *A. niger* and *M. grisea* at 25 °C resulted in a strong increase in protein production.

Table 1
Enzyme activities of single and mixed cultivations at 30 °C (nmol min^{-1} ml^{-1} of culture filtrate). An = *A. niger*, Ao = *A. oryzae*, Pc = *P. chrysosporium*. Combinations with increased activities are in bold.

	An	Ao	Pc	An + Ao	An + Pc	Ao + Pc
β-glucosidase	3.3 ± 0.1	4.0 ± 0.1	2.9 ± 0.4	**4.9 ± 0.1**	**4.0 ± 0.1**	**4.9 ± 0.1**
cellobiohydrolase	3.6 ± 0.2	4.8 ± 0.6	2.3 ± 0.4	4.9 ± 0.2	3.8 ± 0.2	5.5 ± 0.1
β-xylosidase	62.0 ± 0.5	6.6 ± 1.0	4.2 ± 0.1	27.7 ± 0.0	**69.8 ± 0.1**	6.3 ± 0.1
α-arabinofuranosidase	3.3 ± 0.1	3.7 ± 0.3	2.3 ± 0.1	3.4 ± 0.1	3.0 ± 0.1	4.0 ± 0.1
β-galactosidase	23.3 ± 0.1	20.1 ± 0.2	2.0 ± 0.2	19.9 ± 0.4	22.5 ± 0.0	**24.5 ± 0.2**
laccase	1.3 ± 0.1	1.8 ± 0.1	0.2 ± 0.1	1.3 ± 0.0	**3.1 ± 0.2**	**3.6 ± 0.9**

Table 2
Enzyme activities of single and mixed cultivations at 25 °C (nmol min^{-1} ml^{-1} of culture filtrate). An = *A. niger*, Ao = *A. oryzae*, Mg = *M. grisea*. Combinations with increased activities are in bold.

	An	Ao	Mg	An + Mg	Ao + Mg
β-glucosidase	2.4 ± 0.1	1.7 ± 0.1	1.2 ± 0.0	2.3 ± 0.1	**2.8 ± 0.0**
Cellobiohydrolase	1.9 ± 0.1	1.8 ± 0.0	0.6 ± 0.0	**2.4 ± 0.1**	1.9 ± 0.2
β-xylosidase	50.4 ± 0.0	6.5 ± 0.1	2.9 ± 0.1	**52.1 ± 0.1**	**8.2 ± 0.2**
α-arabinofuranosidase	2.6 ± 0.1	2.0 ± 0.1	0.5 ± 0.1	**3.0 ± 0.1**	1.6 ± 0.0
β-galactosidase	20.2 ± 0.1	6.7 ± 0.0	0.8 ± 0.0	**20.7 ± 0.1**	**11.3 ± 0.1**
laccase	1.6 ± 0.3	3.3 ± 0.7	24.1 ± 1.9	**1.8 ± 1.0**	**4.4 ± 0.1**

3.3. Effect of co-cultivation on overall extracellular protein profiles

To study the effect of co-cultivations on overall protein secretion, SDS-PAGE profiles were obtained for all cultivations (Fig. 2). This demonstrated that only in the case of the *A. niger* + *A. oryzae* co-cultivation a combined protein profile of the individual cultures was detected. For the combinations of both *A. niger* and *A. oryzae* with *M. grisea* the profile is highly similar to that of *A. niger* and *A. oryzae* alone. For the combinations of *A. niger* and *A. oryzae* with *P. chrysosporium* protein profiles were obtained containing proteins not observed in the single cultivations.

4. Discussion

The positive effect of co-cultivations of fungi on the production of hydrolytic enzymes has been published previously (Gupte and Madamwar, 1997; Verma and Madamwar, 2002; Zhang et al., 2006). However, no reports have described the effects of co-cultivating the two most important industrial Aspergilli (*A. niger* and *A oryzae*) with each other. In this paper we have addressed this question and also compared the effects of the presence of two other fungi, the plant pathogenic ascomycete *M. grisea* and the basidiomycete wood rot fungus *P. chrysosporium* on these two Aspergilli. The initial growth experiments on plates demonstrated these fungi grew in each other's presence when they are inoculated on the same plate. Three of the four fungi did not grow as mixed colonies, but rather as sectors, but appeared to not compete with each other. In contrast, *P. chrysosporium* appears to grow through the other fungi, again without any apparent competition response. These data and the preliminary experiments with other fungal combinations that did not appear to tolerate each other, suggest that specific fungi are likely to form mixed populations in nature, while others are not likely to actively grow simultaneously. The negative result for *A. niger* and *T. reesei* is surprising as these fungi were reported to be used in co-cultivation for cellulose production (Maheshwari et al., 1994) and might be due to differences in the strains used in our study.

Many of the co-cultivations resulted in increased activity for several enzymes, but often not for all enzymes. This suggests that the co-cultivation does not trigger a general increase in protein secretion but rather induction of specific enzymes, which is confirmed for most co-cultivations by the determination of total extracellular protein. These effects are likely also dependent on the carbon source used. We used wheat bran as a carbon source as earlier studies in our lab showed a broad range of enzymes that are produced using wheat bran as sole carbon source (Meijer et al., in press). The use of an insoluble substrate as wheat bran does not allow for determination of fungal biomass (growth) during the cultivation. However, as most extracellular protein concentrations are in the same range, we expect that overall growth is similar.

Table 3
Total protein production in the single and mixed cultures (mg ml^{-1} of culture filtrate). An = *A. niger*, Ao = *A. oryzae*, Pc = *P. chrysosporium*, Mg = *M. grisea*. ND = not done.

Fungal species	25 °C	30 °C
An	0.20 ± 0.00	0.43 ± 0.02
Ao	0.33 ± 0.02	0.43 ± 0.00
Pc	ND	0.39 ± 0.08
Mg	0.32 ± 0.02	ND
An + Ao	ND	0.38 ± 0.02
An + Pc	ND	0.47 ± 0.10
Ao + Pc	ND	0.53 ± 0.01
An + Mg	0.42 ± 0.01	ND
Ao + Mg	0.33 ± 0.08	ND

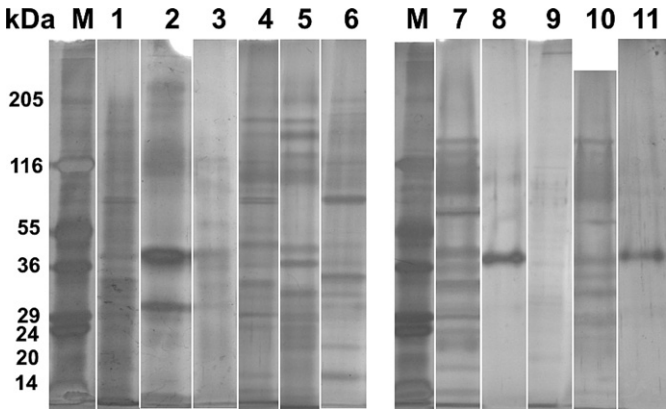

Fig. 2. Protein profiles of separate species and different combinations after growth for 72 h in MM + 1% wheat bran. 1–6: Cultures performed at 30 °C, 7–11: Cultures performed at 25 °C. M. Marker, 1. *A. niger*; 2. *A. oryzae*; 3. *P. chrysosporium*; 4. *A. niger* + *A. oryzae*; 5. *A. niger* + *P. chrysosporium*; 6. *A. oryzae* + *P. chrysosporium*; 7. *A. niger*; 8. *A. oryzae*; 9. *M. grisea*; 10. *A. niger* + *M. grisea*; 11. *A. oryzae* + *M. grisea*.

In some cases the activities of the co-cultivations were above the sum of the single incubations, even though only half the number of spores from each species was used compared to the single incubations. This indicates that the overall activity in the mixed culture is not solely the sum of the individual activities of the fungal species. A similar result was reported for *A. niger* and *T. reesei* with respect to the production of endoglucanase (Maheshwari et al., 1994).

An exception to this was the β-xylosidase activity in the *A. niger* + *A. oryzae* combination that was approximately the average of the two single combinations. This data suggests that both fungi are present in equal amounts in the culture, which is confirmed by the SDS-PAGE profile. This result also stresses even more that co-cultivation can result in specific up-regulation of enzyme production as several other activities in this combination are higher compared to the single incubations.

Another example of the positive effect of one fungus on the production of enzymes by the other is the increase in laccase activity in the combinations of *A. niger* and *A. oryzae* with *P. chrysosporium*. *P. chrysosporium* itself does not produce laccase (Martinez et al., 2004) and therefore the increase in laccase activity must be caused by stimulation of laccase production in *A. niger* and *A. oryzae* by the presence of *P. chrysosporium*.

Very high laccase activity was detected for *M. grisea*, which was strongly reduced in the presence of *A. niger* and *A. oryzae*. As the protein profiles of the mixed cultivation are nearly identical to that of the Aspergilli alone, this suggests that the two Aspergilli are the dominant species in the co-cultivations with *M. grisea*, which could explain this reduction. Why *M. grisea* produces such high laccase activity on wheat bran, especially in comparison to the other enzyme activities is not clear. While laccase activity has been reported previously for *M. grisea* (Iyer and Chattoo, 2003), no studies on the induction of the enzyme have been reported.

This study demonstrates the high potential of fungal co-cultivations for biotechnological applications. This is particularly the case for the co-cultivation of the two industrial fungi, *A. niger* and *A. oryzae*. Future studies will address the mechanism behind the increased enzyme production in co-cultivations of these two fungi. Another topic that remains to be studied is to identify the optimal ratio between the two fungi in a co-cultivation to obtain the best production of enzymes.

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